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13. ABSTRACT (<i>Maximum 200 Words</i>) Vitamin D and its analogs have growth-suppressing and cell-differentiating effects on various carcinoma cell types. We have synthesized a vitamin D analog (1 α (OH)D ₅) that is nontoxic and has both growth-inhibitory and cell-differentiating actions in various established breast cancer cell lines. Our original goal was to covalently link this vitamin D analog to antibody against Her-2/neu protein and thus deliver 1 α (OH)D ₅ specifically to highly aggressive human breast cancer cells. Using the xenograft model, we previously confirmed that 1 α (OH)D ₅ supplemented in the diet inhibits growth of human breast carcinoma cells transplanted into athymic mice. As a second phase of the study, we covalently linked 1 α (OH)D ₅ to Her-2/neu antibody using sulfo-SANPAH linker. The 1 α (OH)D ₅ -Her-2 conjugate specifically binds to Her-2 receptor-expressing breast cancer cells. The conjugate was able to compete with unconjugated Her-2 antibody for Her-2 receptor binding sites on the cancer cells. Our preliminary results show that the conjugate, when injected into Her-2+ tumor-bearing athymic mice, shows significantly higher accumulation in tumor than in other visceral organs. Experiments are in progress to further characterize the properties of 1 α (OH)D ₅ -Her-2 antibody immunoconjugate and its effect on in vivo growth of human breast carcinoma cells.				
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FOREWORD

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Table of Contents

Cover	1
SF 298	2
Foreword	3
Table of Contents	4
Body	5-17

Body of the progress report (1998-99)

♦ Introduction

In recent years, several natural and synthetic agents, especially those with antiproliferative and differentiating properties, have been the primary focus of therapeutic and chemopreventive research. A synthetic analog of vitamin A, N-[4-hydroxyphenyl] retinamide (HPR), is recognized as a chemopreventive agent for breast carcinoma in experimental animals. In addition to vitamin A, Vitamin D has also shown promising results. Vitamin D is classified as a hormone within a steroid hormone family.⁵ It is a secosteroid that is biologically inert until hydroxylated on the carbon 25 position in the liver to form 25-hydroxyvitamin D, which is further metabolized to $1\alpha,25$ dihydroxy vitamin D₃ ($1\alpha D_3$). When it is no longer needed, the hormone gets metabolized to an inactive form (24-hydroxyvitamin D) and excreted from the body.⁶ In addition to its function in maintaining blood calcium level and mobilizing calcium from bone, $1\alpha(OH)D_3$ has growth-suppressing and cell-differentiating actions in many malignant cell types.^{7,8}

One major factor limiting successful use of vitamin D or $1\alpha D_3$ in cancer prevention or therapy is its calcemic activity. The concentration needed to cause reduced growth of neoplastic cells would cause hypercalcemia and death. Therefore, in recent years, attention has been directed to developing analogs that preserve vitamin D's growth suppressive activity but reduce its calcemic activity.⁹ In experimental systems, addition of vitamin D analogs to adriamycin or tamoxifen treatment has shown enhanced growth inhibitory action of the drugs.^{10,11} We recently evaluated a novel vitamin D analog, $1\alpha(OH)D_5$, as a potential antiproliferative or cell-differentiating agent for breast cancer cells. This analog was synthesized by Dr. Robert M. Moriarty, Professor, Department of Chemistry, University of Illinois at Chicago. Table 1 summarizes the results obtained previously in our laboratory. $1\alpha(OH)D_5$ is nontoxic in athymic mice.

♦ Hypothesis

Vitamin D and its analogs have growth-suppressing and cell-differentiating actions in various neoplastic cell types. However, their use as therapeutic or cancer preventive agents is hindered due to their high calcemic activity. We recently studied the effects of a new synthetic vitamin D analog $1\alpha(OH)D_5$ in breast carcinoma cells. This newly synthesized analog appears to have no significant calcemic activity. In experimental systems, $1\alpha(OH)D_5$ inhibited the development of premalignant lesions in DMBA-exposed mammary gland explants. In malignant cells, it induced expression of various markers associated with breast cell differentiation, namely I-CAM, casein, and nm23. Also, altered phenotypic changes were associated with induction of vitamin D receptor (VDR) and TGF β_1 protein. In women, tumors showing overexpression of nm23, ICAM, and e-cadherin are generally noninvasive. In the present study, we hypothesize that $1\alpha(OH)D_5$ treatment could induce breast cancer cell differentiation, render them non-aggressive, and alter their tumorigenicity and metastatic potential. If vitamin D analog proves to induce functional and biological differentiation in breast carcinoma cells, it will be of great value as a chemopreventive agent, particularly in women with premalignant lesions and at high risk of developing aggressive tumor. $1\alpha(OH)D_5$ could be easily given as a dietary supplement. Alternatively, it could be administered at low concentrations as an immunoconjugate with c-erbB2 antibody and specifically targeted for breast carcinoma cells, without any effect on normal cells.

◆ Technical Objectives

$1\alpha(\text{OH})\text{D}_5$ inhibited the development of premalignant lesions in DMBA-exposed mammary gland explants. In malignant cells, $1\alpha(\text{OH})\text{D}_5$ induced expression of various biomarkers (namely ICAM-1, casein, and nm23) associated with breast cell differentiation. Generally, in women, in-situ ductal carcinomas showing overexpression of ICAM-1, nm23, and e cadherine are noninvasive.¹⁶⁻²⁰ Thus, it is likely that induction of various differentiation markers observed following vitamin D_5 treatment may alter functional characteristics of malignant cells, render them nonaggressive, and alter their tumorigenic and invasive potential. In the present study, we aim to evaluate the potential therapeutic and antimetastatic properties of $1\alpha(\text{OH})\text{D}_5$.

- 1) Determine the effects of $1\alpha(\text{OH})\text{D}_5$, a synthetic vitamin D analog, on morphological or phenotypic, functional, and biological characteristics of malignant cells.
- 2) Evaluate therapeutic efficacy of $1\alpha(\text{OH})\text{D}_5$ immunoconjugated with c-erbB2 antibody.
- 3) Study the effects of dietary supplementation of $1\alpha(\text{OH})\text{D}_5$ on growth and metastasis of human breast carcinomas in experimental animals.

Successful completion of the proposed study will identify a new safe, nontoxic chemopreventive and/or therapeutic agent for breast cancer.

◆ Statement of Work for 1998-99

Task 3, 10-15 months: Study effect of dietary supplementation of $1\alpha(\text{OH})\text{D}_5$ in athymic mice. We will study three different cell lines. Each will be injected into 20 animals; 10 animals will receive regular diet, and 10 animals will receive $1\alpha(\text{OH})\text{D}_5$ supplemented diet. A total of 60 animals will be used. Experiments will take 60 days.

Task 4, 16-24 months: We will determine the ability of vitamin D analog-treated cells to form acinar structures, and we will study the effect of $1\alpha(\text{OH})\text{D}_5$ on *in vivo* invasive capacity in matrigel-coated membranes. We will characterize $1\alpha(\text{OH})\text{D}_5$ -c-erbB2 immunoconjugate, determine its binding ability, antibody to drug molar ratio, and *in vivo* distribution of conjugate in various tissues. *In vivo* testing of this conjugate will require at least 60 animals. Tissue concentration of $1\alpha(\text{OH})\text{D}_5$ will be determined by HPLC analysis.

◆ Summary of significant results during last (1997-98) reporting period

- 1) $1\alpha(\text{OH})\text{D}_5$ has growth inhibitory action in selected cells (MCF-7, UISO-BCA-4, BT-474, T-47D, ZR-75-1). Cells incubated with 0.1 to 1 μM $1\alpha(\text{OH})\text{D}_5$ show reduced growth as compared to those incubated without $1\alpha(\text{OH})\text{D}_5$.
- 2) $1\alpha(\text{OH})\text{D}_5$ failed to inhibit *in vitro* growth of MDA-MB-231 cells.
- 3) The effect of $1\alpha(\text{OH})\text{D}_5$ was irreversible. The cells pretreated for 10 days with $1\alpha(\text{OH})\text{D}_5$ (10^{-6}M) failed to achieve a growth rate comparable to untreated cells when returned to normal medium (without $1\alpha(\text{OH})\text{D}_5$).
- 4) Following 7 days *in vitro* exposure of cells (MCF-7, UISO-BCA-4, ZR-75-1) to $1\alpha(\text{OH})\text{D}_5$ (0.1-1 μM), an increase in expression of alpha2 integrin, beta 1 integrin, lipid droplets, ICAM, and casein was noticed as compared to vehicle-treated control cells.
- 5) The effect of $1\alpha(\text{OH})\text{D}_5$ on induction of alpha2 integrin was dose-dependent.

- 6) $1\alpha(\text{OH})\text{D}_5$ failed to show changes in the above-mentioned biomarkers in MDA-MB-231 cells.
- 7) In vivo, $1\alpha(\text{OH})\text{D}_5$, either injected s.c. or supplemented in the diet, reduced growth of ZR-75-1, UISO-BCA-4, and MCF-7 cells transplanted into athymic mice.
- 8) $1\alpha(\text{OH})\text{D}_5$ failed to inhibit the in vivo growth of MDA-MB-231 cells.
- 9) The in vivo growth of ZR-75-1 cells was inhibited by Her-2 antibody given i.p. (5 μg , 3 times weekly). However, ZR-75-1 cells grow in animals only when animals were given supplemental estradiol.

♦ **Progress during the current reporting period**

Effect of $1\alpha(\text{OH})\text{D}_5$ supplemented in the diet in various breast carcinoma cell lines:

Previously we examined in vitro the effects of $1\alpha(\text{OH})\text{D}_5$ in various breast carcinoma cell lines (UISO-BCA-1, ZR-75-1, MCF-7, T-47D, BT-474, MDA-MB-231, MDA-MB-435, SK-BR-3 and MAXF-401). $1\alpha(\text{OH})\text{D}_5$ inhibited the growth of MCF-7, ZR-75-1, UISO-BCA-4, BT-474 and T-47D cells. No significant effect was observed on UISO-BCA-1, MDA-MB-435, and MAXF-401 cells. We also further examined the effect of $1\alpha(\text{OH})\text{D}_5$, $1,25(\text{OH})_2\text{D}_3$, and RO24-5531 on various biomarkers (intracellular lipid droplets, intracellular casein, α_2 integrin, β_1 integrin, ICAM-1, and cytokeratin expression) in these cell types. Amongst all the cell lines studied, we observed induction of one or more differentiation markers in MCF-7, UISO-BCA-4, ZR-75-1, and to some extent in BT-474 cells. Based on these results, we studied the effect of $1\alpha(\text{OH})\text{D}_5$ supplemented in diet in mouse xenograft model. For this study, we used UISO-BCA-4, MCF-7, ZR-75-1 and MDA-MB-231 cells. Results were described in detail in the last report (also see Fig 1a-c). In brief, $1\alpha(\text{OH})\text{D}_5$ inhibited the growth of MCF-7, ZR-75-1, and UISO-BCA-4 cells. No effect was observed on MDA-MB-231 cells. These results clearly suggest that $1\alpha(\text{OH})\text{D}_5$ has growth inhibitory effect in cells expressing VDR. As mentioned in the earlier report, it is evident that most of the cell lines reported to be responsive to $1\alpha(\text{OH})\text{D}_5$ are non-metastatic. $1\alpha(\text{OH})\text{D}_5$ -responsive cells are expressing low or moderate amounts of Her-2/neu. Also, cell lines whose growth is inhibited by $1\alpha(\text{OH})\text{D}_5$ are estrogen-dependent. Estradiol is known to inhibit Her-2 expression. Thus, in order to design breast cancer cell-targeted therapy using Her-2-linked $1\alpha(\text{OH})\text{D}_5$, we need cells that are metastatic in animals, express VDR, are estrogen-independent, and are Her-2-positive. We proposed during the last reporting period to 1) transfect MDA-MB-231 cells with VDR and Her-2/neu and 2) examine the effect of $1\alpha(\text{OH})\text{D}_5$ in metastatic breast lines generated by the PI.

- 1) We have transfected VDR, VDRE, VDR+VDRE, and empty vector in MDA-MB-231 cells. Currently, we are in the process of expanding the selected clones into stably transfected cell lines.
- 2) The effect of $1\alpha(\text{OH})\text{D}_5$ was investigated in two different xenograft lines: UISO-BCA-4met and UISO-NMT-BCA-18. UISO-BCA-4nmt is an original xenograft generated from pleural fluid obtained from a patient with confirmed diagnosis of breast cancer. The UISO-BCA-4 cell line was also originated from the same patient by culturing cells from pleural fluid. UISO-BCA-4 cells are nonmetastatic. Xenografts (UISO-BCA-4met, UISO-NMT-BCA-18 [approximately 50 mg]) were transplanted into 3- to 4-week-old athymic mice. Animals either received control diet or received diet supplemented with $1\alpha(\text{OH})\text{D}_5$ at 12.5 $\mu\text{g}/\text{kg}$ diet.

Figure 1

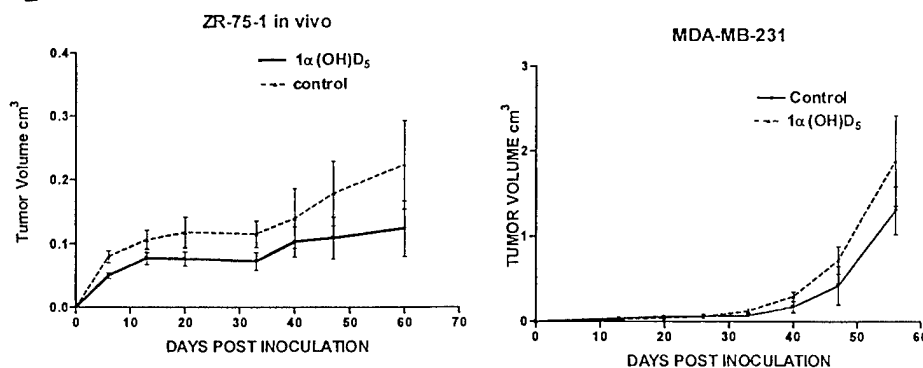
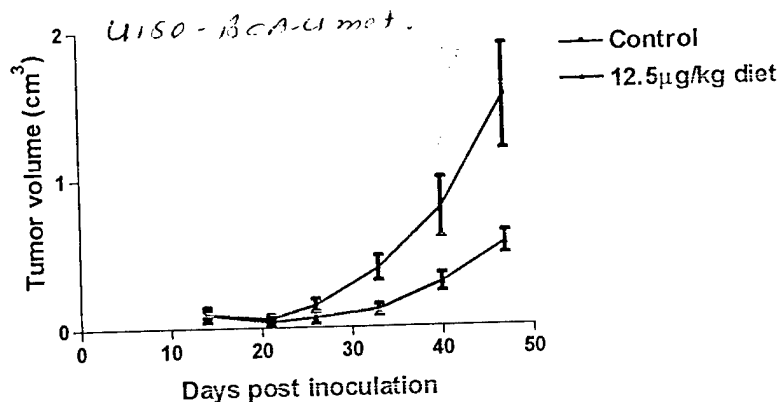


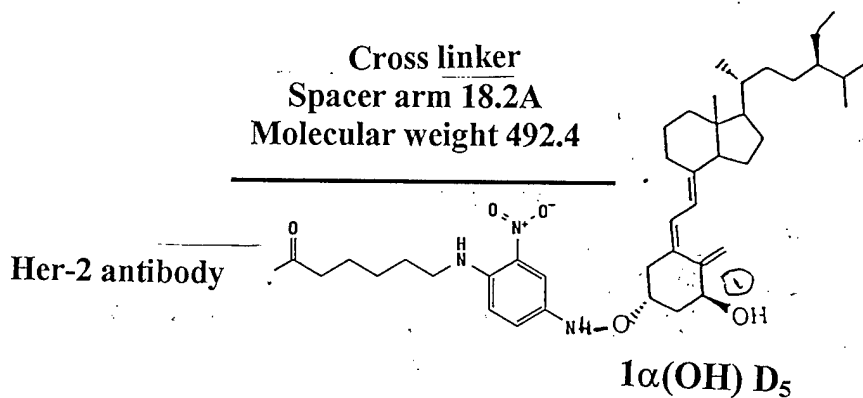
Figure 2



As shown in Fig. 2, in vivo growth of UISO-BCA-4met was significantly inhibited by 1α(OH)D₅. However, we failed to observe apparent metastatic lesions in control or 1α(OH)D₅-treated animals. It is possible that we may have missed micrometastatic lesions in these animals. In UISO-NMT-BCA-18 xenografts, no effect of 1α(OH)D₅ was observed on growth rate.

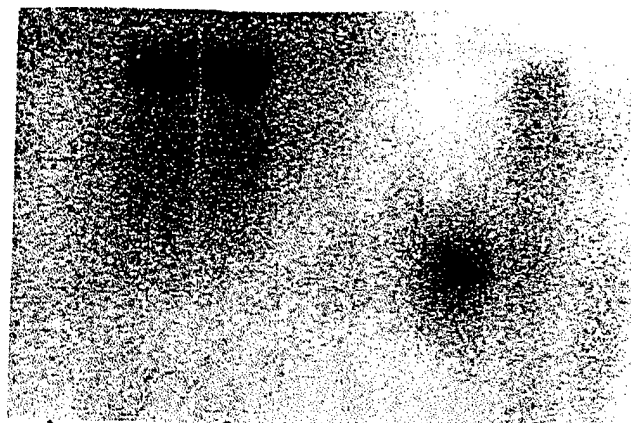
Preparation of 1α(OH)D₅-c-erbB2 antibody conjugate: For linking the Her-2 antibody to 1α(OH)D₅, we used sulfosuccinimidyl 6-[4'-azido-2'-nitrophenylamido] hexanode (Sulfo-SANPAH) as a linker. It has a molecular weight of 492.4 and spacer arm linking the antibody, and 1α(OH)D₅ is approximately 18.2 Å. Antibody against extracellular domain of Her-2/neu (Clone G6.10, Ab-2) was obtained as BSA-free, azide-free solution from Neomarkers (Union City, CA). This antibody is directed against the protein backbone of the extracellular domain of the neu protein. Sulfo-SANPAH cross linker was obtained from (Pierce Biotech Company, Rockford, IL). Conjugation of 1α(OH)D₅ will be performed by a two-stage method based on photoaffinity cross-linking as described by the supplier of the cross linker. Cross linker was initially dissolved in DMSO and then was diluted to different molar concentrations (1-10) in phosphate buffer (pH 7, 0.150M). For initial characterization, we used iodinated neu antibody at different concentrations. Antibody + cross linker solution was first incubated on ice for 60 minutes in the dark. Following incubation, the reaction mixture was dialyzed and then second step reaction was continued. This reaction brings the photoreactive group of the linker face outward from the surface of the modified molecule. In a second step, this photoactivated molecule was coupled by photoactivation to 1α(OH)D₅. Photoactivation was performed by exposing the reaction mixture with 6 bright camera flashes held approximately 2-3 inches above the reaction vessel. The nitro-substituted arylazide group when photolyzed formed an aryl nitrene that can react non-selectively to form a covalent bond. Again, the reaction mixture was dialyzed to remove free vitamin D₅. Fig. 3 shows the structure of 1α(OH)D₅ linked to Her-2 antibody using sulfo-SANPAH cross linker.

Figure 3

Structure of Her-2 antibody linked to $1\alpha(\text{OH})\text{D}_5$ **Identification of $1\alpha(\text{OH})\text{D}_5$ -Her-2 immunoconjugate using SDS page electrophoresis:**

Initially, in order to be cost-effective, preparation of immunoconjugate was tested using monoclonal antibody 19-24, which was generated in our laboratory and is specific for human sarcoma. Fig. 4 shows an autoradiograph of the prepared immunoconjugate (19-24 antibody conjugated to $1\alpha(\text{OH})\text{D}_5$) (lanes 2 and 3) and the radiolabeled antibody alone (lane 6). **We also included on the gel iodinated Her-2 antibodies (lanes 7 and 8; Ab-3 and ab-6 to Her-2/neu, Oncogene Science). The later antibodies were found to be unsuitable for iodination and immunoconjugation; however, ab-2 Her-2 antibody was found to be most suitable for preparation of immunoconjugate (Fig. 5).** We used different molar concentration of linker to determine which concentration shows optimal cross linking with Her-2 neu and $1\alpha(\text{OH})\text{D}_5$. The molar ratios used for linker to IgG were 10:1, 30:1, and 60:1. For initial reaction, $1\alpha(\text{OH})\text{D}_5$ was used at different concentrations (5-25 μl of 10^{-3}M concentration per reaction). Immunoconjugate (2-10 μl) was subjected to SDS polyacrylamide gel electrophoresis using 4-20% gradient gel. Gel was overlaid over photographic X-ray films and exposed for 24 hours. Fig. 5 shows the electrophoretic mobility profile of ^{125}I -labeled c-neu antibody and antibody linked to $1\alpha(\text{OH})\text{D}_5$. The iodinated antibody showed mobility around 150kd molecular weight, whereas the immunoconjugate migrated at a shorter distance, at the position of higher molecular weight. The immunoconjugate appeared to have varying molecular weight (it showed smeared pattern between 150 and 250 Kd or higher molecular weight). In conclusion, the linker-to-antibody ratio (60:1) used in the reaction showed synthesis of immunoconjugate with $1\alpha(\text{OH})\text{D}_5$.

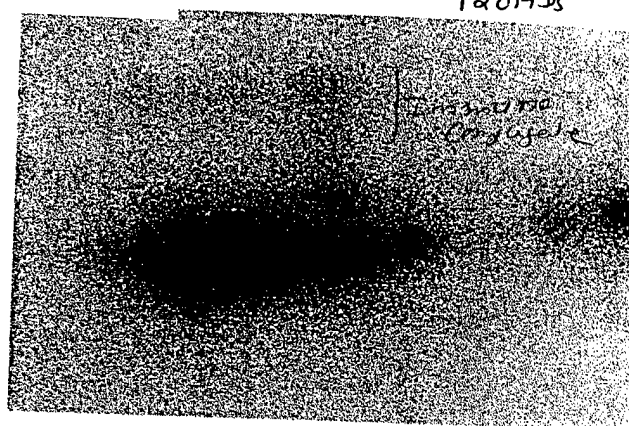
Figure 4



1 2 3
 $1\alpha(OH)D_5$
 Immuno
 conjugate

6 Ab7 8
 19-24 ab-3 ab-5
 antibody along Her-2 ab. Linker
 Ig G. ratio

Figure 5



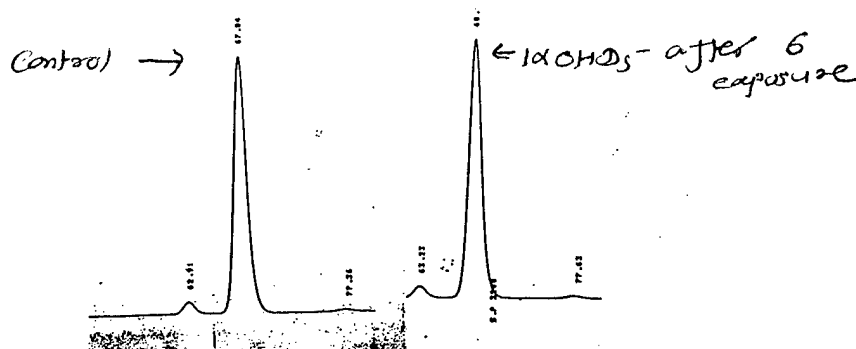
10:1 30:1 60:1

Her-2
 ab

Autoradiogram of immunoprecipitate using different molar ratio of linker to IgG. Her-2 antibody was linked to $1\alpha(OH)D_5$.

$1\alpha(OH)D_5$ was stable after exposure to 3-9 camera flash lights: Generally, compounds structurally related to vitamin D are photosensitive and degrade to a certain extent following exposure to light sources. In order to determine the stability of $1\alpha(OH)D_5$ during camera flash light activation, we made a $1\alpha(OH)D_5$ solution in phosphate buffer and then exposed it 3-9 times to camera flash lights. The reaction mixture was precipitated with sodium acetate buffer and then extracted with methanol and subjected to HPLC analysis. $1\alpha(OH)D_5$ processed in the same manner but not exposed to light was used as an experimental control. As shown in Fig. 6, the camera flash light used for the preparation of $1\alpha(OH)D_5$ -Her-2 linker did not degrade the $1\alpha(OH)D_5$.

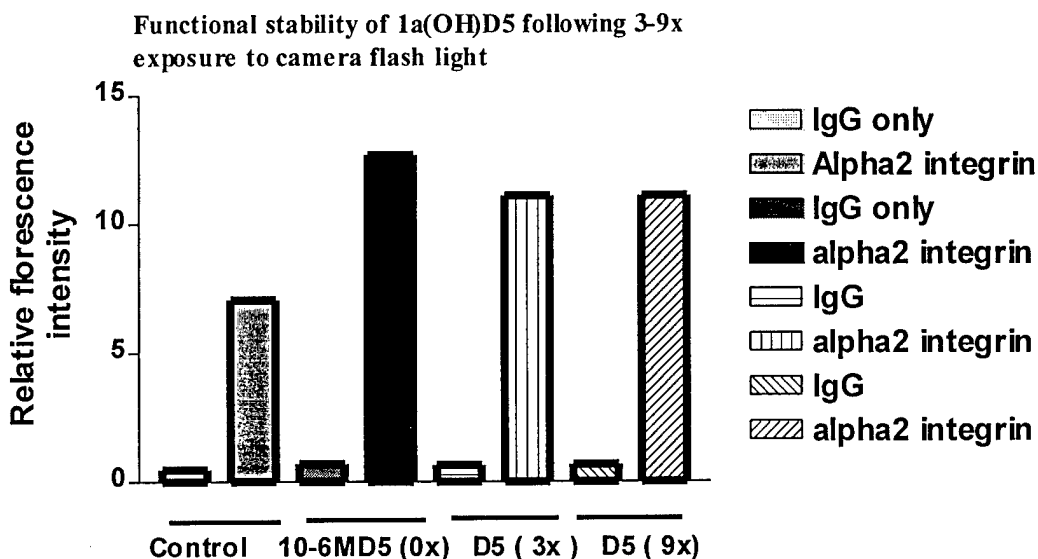
Figure 6



HPLC profile of $1\alpha(OH)D_5$ of control and camera flash exposed (6x) $1\alpha(OH)D_5$.

$1\alpha(OH)D_5$ is functionally active after exposure to light during immunoconjugate biosynthesis: $1\alpha(OH)D_5$ photoexposed for 6 times was functionally active. It increased the expression of alpha2 integrin expression in UISO-BCA-4 cells (results similar to that of authentic $1\alpha(OH)D_5$), when cells were exposed for 7 days at 1 μ M $1\alpha(OH)D_5$ concentration (Fig. 7).

Figure 7

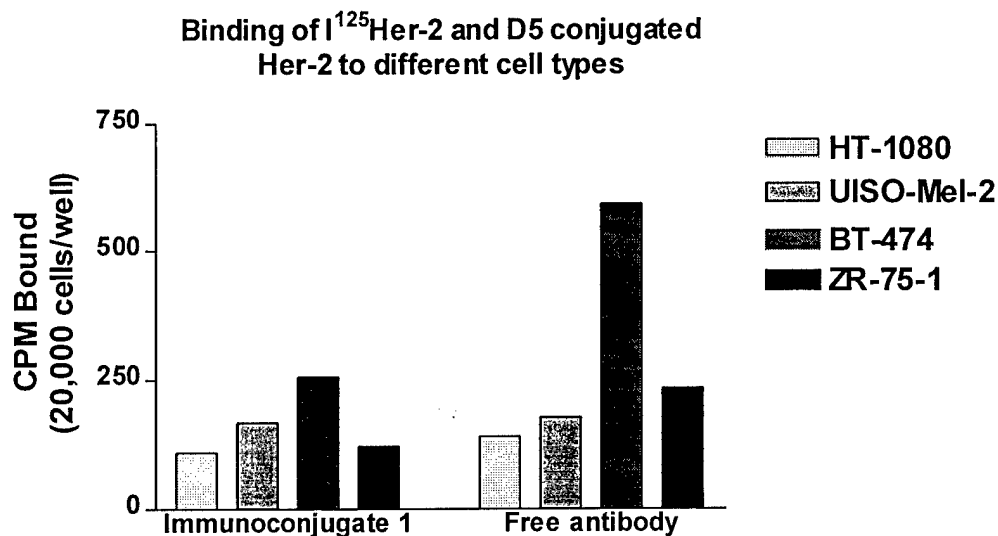


UIISO-BCA-4 cells were incubated with vehicle alone (Control) 10^{-6} M $1\alpha(\text{OH})\text{D}_5$, $1\alpha(\text{OH})\text{D}_5$ exposed 3 times to camera flash light, or $1\alpha(\text{OH})\text{D}_5$ exposed 9 times to camera flash light. Cells were incubated for 7 days. After 7 days, alpha2 integrin expression was determined by FACS analysis. Cells were incubated with either mouse IgG or anti alpha 2 integrin antibody and then FITC-labeled secondary antibody.

$1\alpha(\text{OH})\text{D}_5$ -Her-2 conjugate shows specific binding to Her-2 expressing human breast cancer cells:

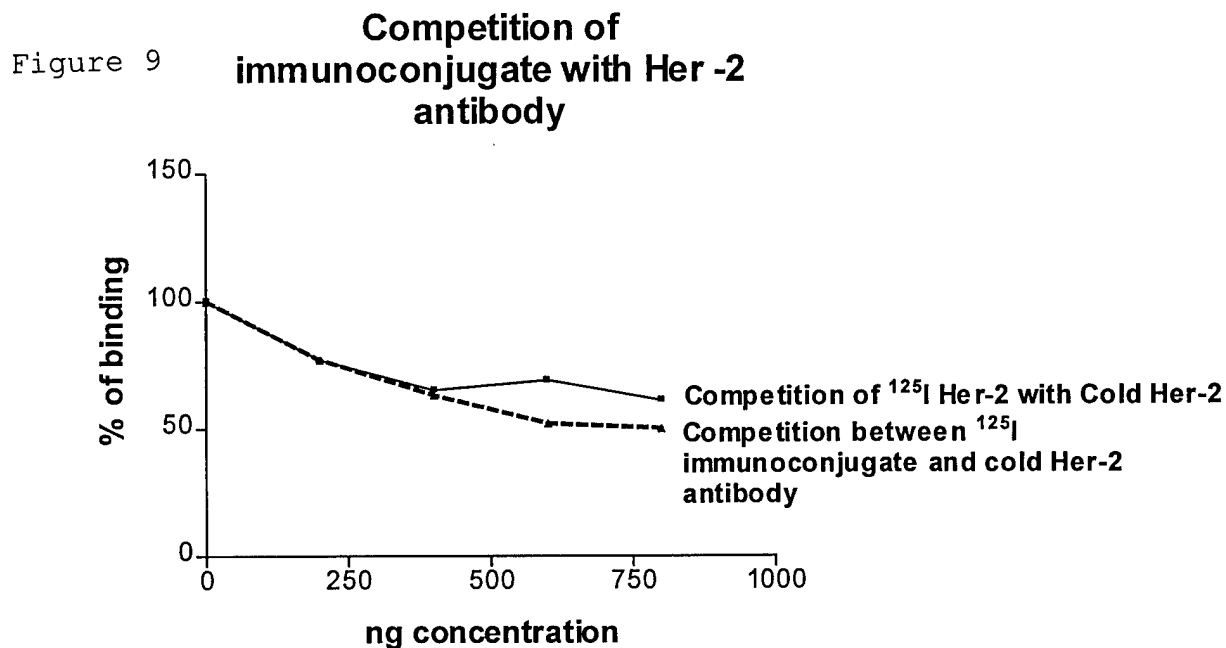
We examined the binding of Her-2 alone and $1\alpha(\text{OH})\text{D}_5$ linked to Her-2 to breast carcinoma cells (BT-474 and ZR-75-1) with different Her-2 status and to other Her-2-negative (HT-1080, UIISO-Mel-2) human cancer cell lines. BT-474 cells are known to show overexpression of Her-2/neu, and ZR-75-1 cells have moderate expression of Her-2/neu. For the binding study, cells (20,000 cells/well) were seeded in 96-well tissue culture plate, and incubated overnight at 37°C in the atmosphere of 5% CO_2 and 95% air. Following 24 hour incubation, media was aspirated, and 0.2 ml of PBS containing 1 million cpm of ^{125}I Her-2 alone or ^{125}I $1\alpha(\text{OH})\text{D}_5$ -Her-2 immunoconjugate was added. Our results are represented in Fig. 8. In cells known to express low or undetectable Her-2, we failed to observe Her-2 or immunoconjugate binding. In BT-474 and ZR-75-1 cells, Her-2 and immunoconjugate binding was evident. Binding of immunoconjugated Her-2 was reduced 47% as compared to that of Her-2 alone in BT-474 cells. These results suggest that, by conjugating $1\alpha(\text{OH})\text{D}_5$, probably some of the binding sites of her-2 are masked, resulting in reduced binding to BT-474 cells. We are currently evaluating whether Her-2 antibody undergoes internalization. If so, we will study what happens to $1\alpha(\text{OH})\text{D}_5$ and whether the compound is still functional and induces VDR in the cells or increases alpha2 integrin expression in the breast cancer cells similar to the effect of authentic $1\alpha(\text{OH})\text{D}_5$.

Figure 8



Various human carcinoma cells (fixed as a monolayer) were incubated with RT for 1 hour with $1\alpha(\text{OH})\text{D}_5$ linked to Her-2 antibody or iodinated Her-2 antibody alone. Radioactivity was counted using a gamma counter.

$1\alpha(\text{OH})\text{D}_5$ -Her-2 conjugate competes with ^{125}I -labeled Her-2 for receptor binding sites in BT-474 cells: In order to determine whether ^{125}I $1\alpha(\text{OH})\text{D}_5$ competes for unlabelled Her-2 antibody for the receptor binding sites, we incubated BT-474 cells with ^{125}I Her-2 antibody in the presence or absence of cold $1\alpha(\text{OH})\text{D}_5$ -Her-2 conjugate or cold Her-2. As shown in Fig. 9, both cold Her-2 antibody and $1\alpha(\text{OH})\text{D}_5$ -Her-2 conjugate were able to compete with Her-2 for receptor binding sites on BT-474 cells.

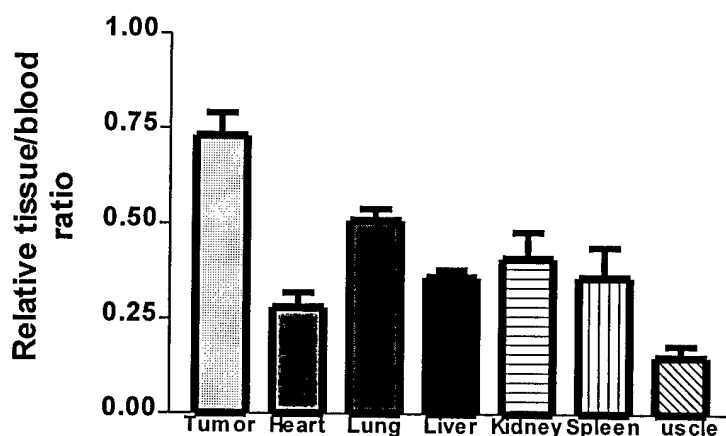


BT-474 cells as a fixed monolayer in 96-well plates were incubated with 1) ^{125}I -Her-2 antibody alone or in the presence of increasing concentrations of cold antibody, 2) with ^{125}I $1\alpha(\text{OH})\text{D}_5$ -Her-2 conjugate alone or in the presence of increasing concentrations of cold Her-2 antibody. Data represent binding of control.

Binding of ^{125}I Her-2 to various tissues in vivo in athymic mice: In order to determine the specific targeting of breast cancer cells with Her-2 antibody, we used athymic the mice xenograft model. Two different human breast carcinoma cell lines (Her-2 positive, BT-474, and Her-2-MDA-MB-231) were used for this purpose. Cells (1 million/animal) were injected into 4- to 6-week-old female athymic mice. Tumors at the site of injection were allowed to attain atleast 1.0 cm diameter in size. Animals were injected i.p. with ^{125}I Her-2 antibody (approximately 2 million cpm). Each group consisted of 6 animals. Animals were sacrificed 24 hours after antibody injection, and accumulation of radioactivity was determined in tumor and other visceral organs. Radioactivity was calculated as ratio of radioactivity per gram of tumor/radioactivity in 1 ml blood. In general, tumors with overexpression of Her-2 (BT-474) had significantly higher (400-fold) accumulation of ^{125}I antibody as compared to those tumors with Her-2 negative tumors from MMDA-MB-231 cells (data not shown). We also observed significant amounts of radioactivity in other visceral organs; however, it was not different between animals with Her-2-negative or Her-2-positive tumors. In the second set of experiment, we injected animals with ^{125}I antibody alone (n=4) or $1\alpha(\text{OH})\text{D}_5$ (n=2) conjugated to ^{125}I Her-2 antibody (approximately 2 million cpm/animal) in animals bearing BT-474 xenografts. Animals were sacrificed 48 hours following injection of the antibody in order to avoid nonspecific radioactivity detection, and radioactivity was counted in preweighed organs using a gamma counter. Fig. 10 shows the distribution of iodinated antibody/ $1\alpha(\text{OH})\text{D}_5$ -linked antibody in the tumor and various organs. Radioactivity is normalized as a ratio of radioactivity in the tissue /radioactivity in the blood. ^{125}I Her-2 antibody showed maximum accumulation in the tumor. Radioactivity was also detected to a lesser extent in spleen, kidney, and lung (Fig. 10).

Figure 10

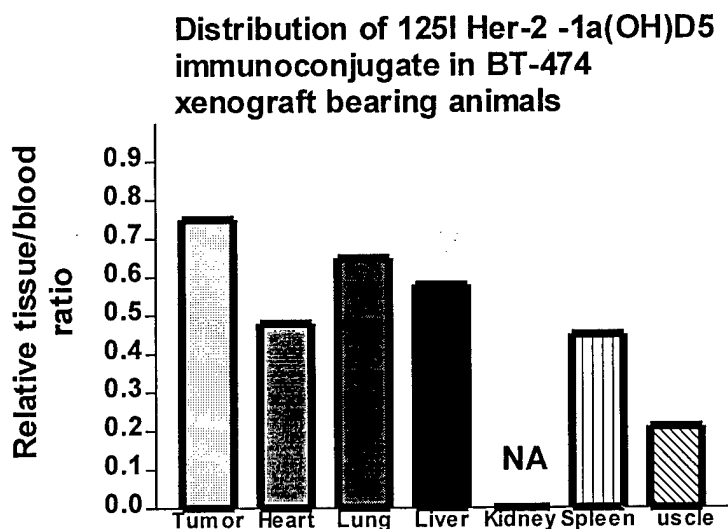
**Distribution of ^{125}I Her-2 antibody
in BT-474 xenograft bearing animals**



Athymic mice bearing BT-474 xenograft tumors were injected i.p. with ^{125}I labeled Her-2 antibody. Animals were sacrificed 48 hours later. Accumulation of radioactivity was determined in tumor and other visceral organs. Data represent ratio of radioactivity observed in gm of tissue to ml of blood.

When the radioactive immunoconjugate was injected into the animals, we found similar distribution of the radioactivity (Fig. 11); however, this experiment needs to be repeated in large groups of animals using fresh immunoconjugate.

Figure 11

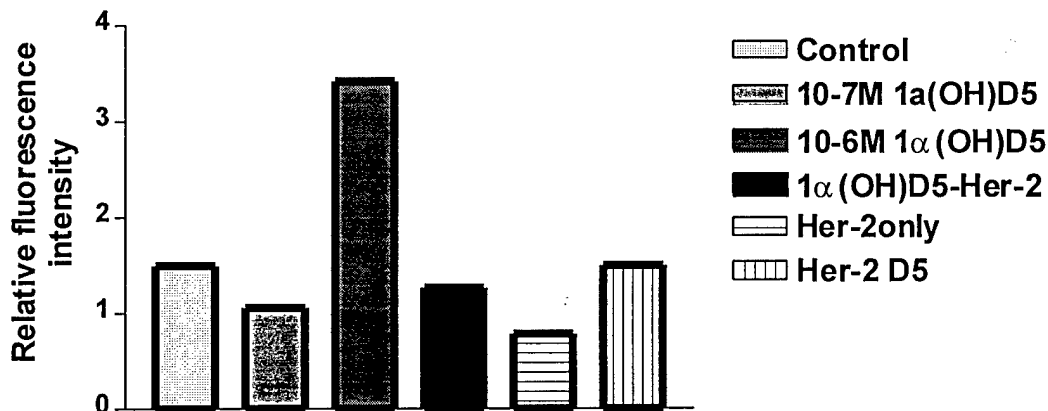


Athymic mice bearing BT-474 xenograft tumors were injected i.p. with ^{125}I -labeled Her-2 antibody linked to 1 α (OH)D₅. Animals were sacrificed 48 hours later. Accumulation of radioactivity was determined in tumor and other visceral organs. Data represent ratio of radioactivity observed in gm of tissue to gm of blood.

Is 1 α (OH)D₅ linked to Her-2 antibody active to induce alpha2 integrin, (or beta 1 integrin) similar to unconjugated 1 α (OH)D₅ in BT-474 cells?: In order to determine whether 1 α (OH)D₅ linked to Her-2 antibody is functionally active to induce breast cancer cell differentiation, we determined alpha2 integrin expression in BT-474 cells following 7 days incubation with vehicle alone, 1 α (OH)D₅ alone, 1 α (OH)D₅ linked to Her-2, Her-2 antibody alone or Her-2 +D₅ added separately. Alpha2 Integrin expression was enhanced significantly by 1 μM 1 α (OH)D₅ treatment but was not significantly changed by 0.1 μM 1 α (OH)D₅ treatment. On the other hand, cells incubated with Her-2 antibody showed reduced alpha2 integrin expression. When cells were incubated with immunoconjugate, they displayed alpha2 integrin levels similar to those observed in control cells. Also, the effect of immunoconjugate was similar to that of cells incubated in combination of both Her-2 +10⁻⁷M D₅, added separately (Fig. 12). These results suggest that Her-2 antibody reduces the expression of alpha2 integrin and that 1 α (OH)D₅ conjugated to Her-2 is still functional and increases alpha2 integrin expression to control levels.

Figure 12

alpha 2 expression in BT-474 cells (9/15/99)



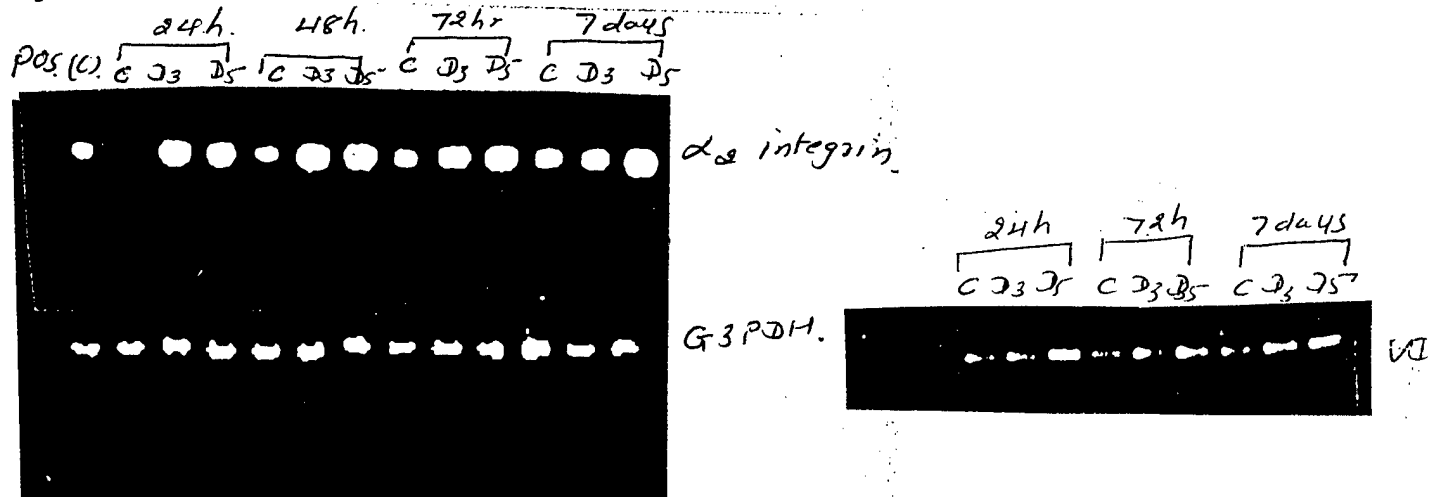
The effect of Her-2/neu, 1 α (OH)D₅, or conjugate on expression of alpha2 integrin in BT-474 cells-FACS analysis.

Experiments related to molecular action of 1 α (OH)D₅ in breast cancer cells.

Previously, we observed enhanced expression of alpha2 integrin, beta 1 integrin, and VDR in human UIISO-BCA-4 breast cancer cells. We further confirmed using RT-PCR that the effect of 1 α (OH)D₅ was at the molecular level, we observed increased expression of alpha2 integrin m-RNA 24 hours following 1 α (OH)D₅ treatment. Similar results were obtained for VDR expression by RT-PCR analysis. For loading control, we determined G3-PDH in the same samples (Fig. 13).

Currently, we are examining expression of alpha2 and beta 1 proteins in human breast cancer cells. Because we previously observed increase in alpha2 integrin levels by immunohistochemistry and by FACS analyses after 7 days treatment, we first evaluated this time point. By western blot analysis, we observed increased expression of alpha2 integrin in D3- and D5-treated cells following 7 days exposure. Whether increases in these protein levels appear at an earlier time point is not certain, and experiments are still in progress.

Figure 13



We are also confirming the effect of $1\alpha(\text{OH})\text{D}_5$ on other commercially available cell lines.

◆ **Future planning of experiments:**

- 1) Standardization of method for determination of $1\alpha(\text{OH})\text{D}_5$ in the immunoconjugate
- 2) Determine whether Her-2 antibody linked to $1\alpha(\text{OH})\text{D}_5$ is still show internalization
- 3) Determine whether $1\alpha(\text{OH})\text{D}_5$ linked to Her-2 antibody is still functional to induce VDR, alpha2 m-RNA in breast cancer cells.
- 4) Determine why Her-2 antibody reduces alpha2 integrin expression in breast cancer cells
- 5) Repeat biodistribution of immunoconjugate in tumor-bearing animals
- 6) Determine whether $1\alpha(\text{OH})\text{D}_5$ conjugated to Her-2 antibody influences growth of breast cancer xenografts in athymic mice.

◆ **Key Research Accomplishments:**

- ◆ $1\alpha(\text{OH})\text{D}_5$ showed cell-differentiating actions in breast cancer cells. The effect of the compound was at a molecular level.
- ◆ $1\alpha(\text{OH})\text{D}_5$ showed growth inhibitory action in selected breast cancer cell lines (both in vivo and in vivo).
- ◆ We were successful in linking $1\alpha(\text{OH})\text{D}_5$ to Her-2 antibody using sulfo-SANPAH
- ◆ $1\alpha(\text{OH})\text{D}_5$ linked to Her-2 antibody showed specific binding to Her-2-expressing breast cancer cells and was able to compete with Her-2 antibody for Her-2 binding sites on breast cancer cells.
- ◆ $1\alpha(\text{OH})\text{D}_5$ did not show degradation during exposure to photoactivation during the conjugation process.
- ◆ $1\alpha(\text{OH})\text{D}_5$ is functionally active to induce alpha2 integrin in breast cancer cells; results were similar to authentic $1\alpha(\text{OH})\text{D}_5$.
- ◆ Even though experiments were performed in small groups of animals, both Her-2 antibody and $1\alpha(\text{OH})\text{D}_5$ linked to Her-2 appear to accumulate mainly in Her-2+ breast tumors when injected into xenograft-bearing athymic mice.

Reportable Outcomes:

Manuscript accepted for publication:

- 1) Mehta R.R., Bratescu L., Graves J.M., Green A., Mehta R.G. Differentiation of human breast carcinoma cells by a novel vitamin D analog: 1α -hydroxyvitamin D₅. Int. J Oncology, 1999, In press

Presentation at meeting

- 1) Mehta R.R., Graves J.M., Mehta R.G. Induction of alpha2 integrins during differentiation of breast cancer cells by $1\alpha(\text{OH})\text{D}_5$. Proc. Am. Assoc. Cancer Res. Vol. 40, pp 650, 1999

Funding applied for based on work supported by this award

- 1) U.S. Army Breast Cancer Translational Research Grant, $1\alpha(\text{OH})\text{D}_5$ as a chemotherapeutic or possibly chemopreventive agent, PI: Das Gupta T.K., Co Investigator, R.R. Mehta, funded
- 2) NCI, Vitamin D₅ in prevention and treatment of breast cancer. , PI: R.G. Mehta (R.R. Mehta Co-I), Pending

◆ **Conclusions:**

- ◆ Results obtained from our study clearly suggest that $1\alpha(\text{OH})\text{D}_3$ is a potent cell-differentiating and antiproliferative agent. Using Her-2 linked $1\alpha(\text{OH})\text{D}_3$, breast cancer cells could be targeted specifically without causing toxicity.

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Sept 28, 1999.

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Dear Sir,
Enclosed please find an original and two copies of annual report of my grant N0. DAMD17-97-1-7263. If you need additional information please feel free to contact me at (312) 996-3692.

Sincerely



R. R. Mehta
Research Associate Professor
Department of Surgical Oncology,

UIC